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Emerging Role of the Gut Microbiome in Nonalcoholic Fatty Liver Disease: From Composition to Function

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Abstract

The gut microbiome, a diverse microbial community in the gastrointestinal tract, plays a pivotal role in the maintenance of health. The gut microbiome metabolizes dietary and host-derived molecules to produce bioactive metabolites, which have a wide array of effects on host metabolism and immunity. 'Dysbiosis' of the gut microbiome, commonly considered as perturbation of microbiome diversity and composition, has been associated with intestinal and extra-intestinal diseases, including nonalcoholic fatty liver disease (NAFLD). A number of endogenous and exogenous factors, such as nutritional intake and xenobiotic exposure, can alter the gut microbiome. We will review the evolving methods for studying the gut microbiome and how these profiling techniques have been utilized to further our understanding of the gut microbial community composition and functional potential in the clinical spectrum of NAFLD. We will highlight microbiome-host interactions that may contribute to the pathogenesis of NAFLD, with a primary focus on mechanisms related to the metabolic output of the gut microbiome. Finally, we will discuss potential therapeutic implications of the gut microbiome in NAFLD.

Keywords

Microbiota; Nonalcoholic Fatty Liver

The gut microbiota is a diverse microbial community comprised of bacteria, fungi, viruses, and archaea that encodes several orders of magnitude more functional genes than the human genome.[1] The collective genetic material of the microbiota is often referred to as the "gut microbiome" and encodes pathways that produce a wide array of bioactive small molecules that are derived from dietary or metabolic precursors and may alter human health.[1] While under normal circumstances, the relationship between the human host and gut microbiome is

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mutually beneficial, perturbations of the gut microbiome, often referred to as “dysbiosis,” have been associated with a number of chronic diseases, including obesity, metabolic syndrome, and nonalcoholic fatty liver disease (NAFLD).[1]

Pre-clinical studies have provided the strongest evidence for a causal role of the gut microbiome in NAFLD. Several pivotal studies established that mice lacking gut microbiota are resistant to the development of diet-induced hepatic steatosis and that hepatic steatosis is transmissible via fecal microbiota transplantation (FMT) and ameliorated by probiotics and antibiotics in murine models.[2] More recent studies suggest that the manipulation of the gut microbiome, either with antibiotics or FMT, also suppresses liver tumorigenesis and reduces portal hypertension in murine models.[3, 4]

Given this compelling pre-clinical evidence, the gut-liver axis is a rapidly developing area of investigation and new insights are emerging from a growing number of human studies. This review will highlight current methods for studying the microbiome and human gut microbial profiles associated with the clinical spectrum of NAFLD, stratified by community composition and function. We will also review postulated mechanisms linking the gut microbiome to the pathogenesis of NAFLD. Finally, we will discuss potential therapeutic implications of the gut microbiome in NAFLD.

Methods for gut microbiome profiling

Advances in profiling and analytic techniques are transforming microbiome research and have been recently reviewed elsewhere[5, 6], so we will limit our discussion to an overview of methods that have been utilized in human studies in NAFLD. (Figure 1) To date, the majority of studies have utilized culture-independent, biomarker-based profiling techniques. This method involves sequencing a ubiquitous gene, which is represented by the 16S ribosomal RNA (16S rRNA) gene in bacteria. Biomarker-based profiling techniques provide a relatively accurate fingerprint of microbial community composition (i.e. taxonomic relative abundance); however, little can be learned about the microbial community’s functional properties.[1] While inferential algorithms based on reference genome databases enable predictions of functional capacities from 16S rRNA sequences, there are limitations to functional predictions.[1] Moreover, this sequencing approach lacks the resolution needed to identify bacteria on a species or strain level, and different strains of the same bacterial species can exert different effects on the human host.[5]

Recent advances in computational biology have improved the feasibility of systems-level “omics” approaches, which allow for microbial community characterization beyond compositional states.[5, 6] These approaches include next generation sequencing approaches to determine the functional genes encoded (metagenomics) or expressed (metatranscriptomics) by a microbial community, and mass spectrometry platforms to identify proteins (metaproteomics) and bioactive small molecules (metabolomics) collectively produced by a microbial community.[1]

Shotgun metagenomic sequencing characterizes the DNA library from a microbial community to obtain the entire gene complement (“metagenome”), although this method

cannot assess the activity of microbial gene expression, which is regulated at the transcriptional and translational level.[1] Even with metagenomic sequencing data, predicted function should be interpreted with caution because pathway presence does not reveal information about activity or directionality. Nevertheless, when compared to biomarker-based sequencing, metagenomics allows for more accurate characterization of microbial functional properties, in addition to taxonomical resolution to the species level.[1] Metabolomics facilitates the identification and quantification of small molecule metabolic products ("metabolome") through use of complementary analytical chemistry techniques and can include both targeted and untargeted approaches.[1]

For the purpose of this review, we will use the term "functional potential" to represent the gene content and/or metabolic output of the gut microbiome, as measured by one or more "omic" approaches.

Human gut microbiome profiles in clinical phenotypes of NAFLD: community composition

Numerous human studies have demonstrated an association between gut dysbiosis and the spectrum of NAFLD in children[7–10] and adults[11–23]. All except one of these studies were cross-sectional[12], and the majority utilized biomarker-based sequencing to profile the gut microbiome. We will review gut microbiome profiles, with a focus on genus-level differences, in the following clinical phenotypes: nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), NAFLD-related advanced fibrosis, and NAFLD-related hepatocellular carcinoma (HCC) (Table 1).

NAFL

Studies comparing the bacterial taxonomic composition between patients with NAFL and controls have yielded variable and often contradictory findings.[8–11, 15, 16, 18, 21, 23] A common finding in NAFL patients is an increase in *Lactobacillus* and *Escherichia* and decrease in *Coprococcus*. However, studies have demonstrated contradictory findings in the relative abundance of *Ruminococcus*, *Prevotella*, and *Bifidobacterium*. Only one study has evaluated the gut microbiota in non-obese adults and found a decrease in *Lactobacillus*[15], contrary to findings in obese NAFL.

NASH

Although more consistent findings have been reported in studies comparing microbial composition in NASH with NAFL and/or controls, there is still discrepancy in findings.[7, 9, 10, 12–14, 16, 18, 19] Several studies found that patients with NASH had a decreased abundance of *Faecalibacterium*[12, 18] and *Ruminococcus*[18], whereas NASH was associated with an increase in *Ruminococcus* in a separate study[9]. Meanwhile, Zhu *et al.* noted a significant difference only in *Escherichia*, which was not appreciated in other studies.[7] To date, only one study has examined the gut microbiota in non-obese adults and noted a significant reduction in *Faecalibacterium*[19], which is consistent with findings in obese NASH.[12, 18] However, *Lactobacillus* and *Ruminococcus* were reduced in non-obese NASH,

similar to findings in non-obese NAFL, and consistent with findings that these genera may have a role in weight regulation.[24, 25]

NAFLD-related advanced fibrosis

NAFLD-related advanced fibrosis (stage>2) is associated with an overall decrease in microbial diversity, secondary to an increase in gram-negative bacteria.[14, 16, 17, 20, 22] Multiple studies have identified associations between *Bacteroides* [14, 17, 20, 22] and *Escherichia*[16, 17, 22] and advanced fibrosis. Similar perturbations of the gut microbiome have been noted in adults with cirrhosis attributable to etiologies other than NAFLD.[2] Surprisingly, only one study identified a reduction in *Akkermansia*, despite evidence that *Akkermansia muciniphila* is associated with other metabolic diseases.[26, 27]

Loomba *et al*. utilized shotgun metagenomic sequencing, thus allowing for species level resolution, and noted that *Bacteroides vulgatus* and *Escherichia coli* were the most abundant species in patients with NAFLD-related advanced fibrosis.[17] This finding is concordant with a prior observation that *Bacteroides vulgatus* is a contributor to insulin resistance.[28] Due to utilization of 16S rRNA sequencing in other human studies examining advanced fibrosis, comparisons with these species-level findings are not feasible.

NAFLD-related HCC

Human studies examining the role of the gut microbiota in NAFLD-related hepatocarcinogenesis are lacking. Ponziana *et al*. were the first to examine adults with NAFLD-related cirrhosis with and without HCC, as compared to healthy controls.[20] *Bacteroides*, *Oscillospira*, and *Enterococcus* were more abundant in HCC, when compared to cirrhosis without HCC.[20] Only one other human study has profiled the gut microbiota in HCC (not limited to NAFLD-related HCC) and found higher levels of *Escherichia coli* through a culture-based approach.[29]

Limitations of compositional studies

Contradictory results between compositional studies could be attributed to various study-specific factors such as differences in patient cohorts (e.g. age, race/ethnicity, geographic location, body mass index [BMI]), definition of fatty liver disease (lack of histopathologic diagnosis in some studies), and comparison groups. Moreover, the majority of studies did not measure or adjust for endogenous or exogenous factors that are known to influence the gut microbiome. Although the gut microbiome is comprised of a stable ‘core’, a dynamic component exists that can be influenced by host and environmental factors.[1] Age, host genetics, sex and hormonal cycles, diurnal variation, geographic location, illness, physical activity, xenobiotic exposure (including antibiotics), and nutritional intake may all impact the gut microbiome.[1] Prior studies have identified country-specific microbial signatures, suggesting that geography and culture (including diet) significantly impact the gut microbiome.[30] While antibiotic treatment is known to alter the gut microbiome[1], we are increasingly recognizing that non-antibiotic drugs also modulate the gut microbiome. For example, a recent study examined 1,000 non-antibiotic drugs against 40 bacterial strains, and found that 24% of drugs with human targets inhibited growth of at least one strain *in vitro*.[31] These results are consistent with studies that have demonstrated microbiome

alterations associated with the use of common medications such as proton pump inhibitors[32] and metformin[33]. While it remains unknown to what degree each of these factors modulate the microbiome-host interaction in NAFLD, these potential confounders must be measured in human studies.[5]

Of note, all studies to date in patients with NAFLD have focused exclusively on bacterial communities, and nothing is known of changes in co-existing fungal and viral communities. Although bacteria dominate the gut microbiota, fungal and viral communities are increasingly recognized as integral members of the community, and trans-kingdom interactions are likely to be in part responsible for ecological balance.[1, 34] Perturbations in fungal community composition have now been associated with several chronic diseases, including obesity[35], and warrants further investigation in NAFLD.

Ultimately, gut microbiome studies in larger, well-characterized, multi-ethnic, international cohorts are needed to better profile the gut microbiota in the clinical spectrum of NAFLD. However, as opposed to the presence of a single microbial species or pathogenic microbiome as the mediator of NAFLD, it's plausible that several pathogenic microbiome states exist and exert differential effects on the human host based on environmental factors and/or genetic susceptibility for NAFLD. For example, a re-analysis of combined data from gut microbiome studies in obesity refuted prior claims that obesity is associated with one unique taxonomic signature.[36] Nonetheless, changes in composition are likely not as important as changes in the *functional potential* of the gut microbial community. It is increasingly recognized that the metagenome encodes substantial redundancy among microorganisms and that there is incongruence between species abundance and transcriptional activity.[37] This was exemplified in a study of lean and obese individuals who were better differentiated based on their gut metagenome, as opposed to their taxonomic profile.[38]

Human gut microbiome profiles in clinical phenotypes of NAFLD: functional potential

Several human studies have employed "omics" techniques and thus added new perspectives on functional attributes of the gut microbiome in NAFL, NASH, and NAFLD-related advanced fibrosis.[7–9, 11, 13, 17, 18, 21, 22] Results from fecal and serum metabolite profiling are listed in Table 1. To date, no human studies have functionally profiled the gut microbiome in NAFLD-related HCC.

NAFL

Two studies have performed integrated analyses of the gut microbiota via 16S rRNA sequencing and targeted metabolomics for fecal metabolites in adults with NAFL. Raman *et al.* identified 18 fecal metabolites that were differentially abundant in obese adults with NAFL, when compared to healthy controls. The majority of differentially abundant fecal metabolites were esters of short-chain fatty acids (SCFAs), including propanoic acid and butanoic acid.[11] Da Silva *et al.* performed targeted profiling of eight fecal metabolites of interest and found that higher concentration of two fecal SCFAs, propionate and isobutyric

acid, differentiated adults with NAFL from healthy controls, corroborating findings from Raman *et al.*[18]

Hoyles *et al.* performed an integrated analysis of the gut metagenome, hepatic transcriptome, and serum and urine metabolomes in a cohort of obese, non-diabetic women.[21] NAFL was associated with low microbial gene richness and alterations in branched-chain and aromatic amino acid pathways and endotoxin synthesis. While multiple microbial-derived metabolites were correlated with NAFL, serum phenylacetic acid, a product of amino acid metabolism, had the strongest association.

NASH

Del Chierico *et al.* identified increased abundance of two serum metabolites, 2-butanone and 4-methyl-2-pentanone, in children with NASH.[39] When combined with taxonomic differences (decreased *Oscillospira* and increased *Dorea* and *Ruminococcus*), these fecal metabolites differentiated children with NASH from healthy controls. Intriguingly, 2-butanone was also increased in the serum of adults with NAFL[21], but the functional significance of this metabolite is unknown.

NAFLD-related advanced fibrosis

Loomba *et al.* performed shotgun metagenomic sequencing in adults with NAFLD (with a focus on NAFLD-related advanced fibrosis), which was integrated with serum metabolomics.[17] Thirty-seven bacterial species, of which *Escherichia coli* was the most abundant, were differentially represented in the gut of patients with advanced fibrosis, compared to those without fibrosis. When these species were incorporated into a model that also included patient age, BMI, and a microbial diversity index, the model possessed high accuracy for detecting advanced fibrosis. However, the differential abundance of serum metabolites and gut microbial gene pathways between groups did not achieve statistical significance. A more recent study by Caussy *et al.* identified a shared genetic determination of serum 3-(4-hydroxyphenyl)lactate, a microbial-derived metabolite involved in amino acid metabolism, with NAFLD-related fibrosis.[22] Interestingly, 3-(4-hydroxyphenyl)lactate was strongly correlated with the abundance of 7 bacterial species that were previously associated with advanced fibrosis, including *Bacteroides caccae*, *Escherichia coli*, and *Clostridium sp.* [17] Moreover, the finding of dysregulation in amino acid metabolism is consistent with metagenomic shifts noted in NAFL.[21]

Postulated mechanisms linking the gut microbiome to NAFLD

Although human studies have yielded insight into functional attributes of the gut microbiota in NAFLD, much of the mechanistic evidence linking the gut microbiome and NAFLD pathogenesis has been obtained from experiments in animal models. We will summarize the current evidence for postulated microbiome-associated mechanisms contributing to the pathogenesis of NAFLD (Figure 2).

1. Altered gut barrier function, endotoxemia, and activation of toll-like receptor mediated pathways

Impairment of the gut barrier, predominantly caused by disruption of intracellular tight junctions, is more common in adults with NAFLD and can even occur in healthy subjects transitioned to a Western diet.[40, 41] Intestinal epithelial barrier disruption leads to increased translocation of microbial products such as lipopolysaccharide (LPS) into the portal circulation, and resultant endotoxemia can induce hepatic inflammation. Rahman *et al.* demonstrated that administering a high fat, fructose and cholesterol diet to knockout mice for the gene encoding junctional adhesion molecule A resulted in severe fibrotic steatohepatitis compared to only modest steatosis in control mice, and administration of oral antibiotics or sequestration of bacterial endotoxins resulted in improvement of liver histology.[42] While impairment in gut permeability appears to contribute to NAFLD pathogenesis, it remains unclear if patients with NAFLD are predisposed to altered gut barrier function, if dietary changes directly affect intestinal permeability, or if a Western diet leads to deleterious changes in the microbiota that mediate impairments in gut barrier function.[43]

Interestingly, knockout mice for mucin 2, with a resulting decrease in intestinal mucus, appear to be protected from high-fat diet induced NAFLD mediated by an increase in IL-22, suggesting an important relationship between the intestinal immune system, the gut barrier, and NAFLD.[44] Modulation of the gut immune system in beta 7 integrin-deficient mice through use of 5-aminosylicylclic acid as a topical anti-inflammatory improved metabolic parameters and reduced gut permeability and endotoxemia.[45] Downstream effects of endotoxin translocation may include induction of toll-like receptors (TLR) in the liver, specifically TLR4, with downstream activation of transcription factors inducing an inflammatory response, and TLR4 knockout may mitigate hepatic inflammation.[46] However, a recent phase 2 clinical trial demonstrated no benefit from a TLR4 antagonist in patients with biopsy-proven NASH, and the ability to manipulate this pathway to ameliorate NAFLD remains uncertain.[47]

2. Choline metabolism

The link between reduced choline bioavailability and the development of NAFLD was established decades ago.[48] Choline deficiency leads to abnormal phospholipid synthesis, defective very-low-density lipoprotein secretion, and alterations in the enterohepatic circulation of bile acids.[49] A number of factors affect choline bioavailability, including dietary intake, estrogen status, and single-nucleotide polymorphisms (SNPs) in genes for *de novo* choline metabolism.[49] However, it was more recently discovered that dietary choline is metabolized by the gut microbiome to produce a variety of metabolites such as trimethylamine (TMA), and thus can reduce choline bioavailability.[50]

At least 8 human gut microbes are avid choline metabolizers, and only low levels of colonization with TMA-producing species are required to significantly reduce host choline levels.[51, 52] A high-fat diet leads to an increase in gut microbes that metabolize choline and subsequent development of hepatic steatosis in mice.[53] Manipulation of dietary intake of choline in human subjects resulted in variations in the abundance of *Gammaproteobacteria*

and *Erysipelotrichi*, which were directly associated with the degree of liver fat accumulation during periods of choline depletion. Abundance of these two classes of bacteria, along with SNPs in choline metabolism, accurately predicted the degree to which subjects developed hepatic steatosis while on a choline-deficient diet.[54]

TMA diffuses into the bloodstream and is metabolized by the liver to generate trimethylamine-*N*-oxide (TMAO). Elevated circulating TMAO levels have been associated with cardiovascular disease[55] and chronic kidney disease[33]. Intriguingly, these are extrahepatic manifestations of NAFLD, which is suggestive of a microbialderived mechanistic link between inter-related cardiometabolic diseases. However, the association between circulating TMAO and NAFLD, in addition to specific microbe-host interactions, is not well delineated.

3. Production of short-chain fatty acids

Indigestable carbohydrates (e.g. dietary fiber) undergo fermentation by the gut microbiota and give rise to SCFAs, which are amongst the most abundant of microbialderived metabolites. The Bacteroidetes phylum is a major contributor to the production of acetate and propionate, whereas the Firmicutes phylum predominantly produces butyrate.[56] While SCFAs provide the majority of energy needs for intestinal epithelial cells, they also cross the intestinal epithelial barrier and mediate a diverse array of biological activities, including regulation of energy expenditure, appetite, and satiety hormone production, through G-protein coupled receptors in multiple tissue sites.[57, 58]

Overweight adults, as compared to lean adults, have increased total gut SCFAs.[59] Additionally, total gut SCFAs decrease in obese adults with anti-obesity treatment and prebiotic administration.[60, 61] Altogether, these data suggest that increased energy extraction from dietary intake, manifested by an increase in SCFAs, is a hallmark of the obesity phenotype. However, in contrast to these findings, increasing colonic propionate prevents weight gain in overweight adults[62], and the beneficial metabolic effects of FMT from lean donors to obese men was associated with an increased abundance of butyrate-producing gut microbes.[63]

Supplementation of SCFAs improves diet-induced hepatic steatosis in murine models[64]; however, in contrast to these findings, human studies have noted increased fecal concentrations of SCFAs in adults with NAFL.[11, 18] (Table 1) In both obesity and NAFLD, incongruous findings for the association between clinical phenotypes and SCFAs are likely attributable to the differential abundance of individual SCFAs, each of which may have different effects on host metabolism.

4. Metabolism of bile acids

Bile acids are increasingly recognized as important signaling molecules that activate a number of host receptors, including farnesoid X receptor (FXR), with effects on host metabolism and immunity.[65] Bile acids prevent intestinal bacterial overgrowth, both directly (via membrane damaging effects) and indirectly (via production of antimicrobial proteins). As such, bile acids modulate the composition of the gut microbiome. On the other hand, the gut microbiota deconjugate and convert primary bile acids to secondary bile acids, thus

regulating the bile acid pool.[66] While a wide array of gut microbes have the capacity to deconjugate bile acids, only small number of bacteria (predominantly *Clostridium* species) facilitate conversion of primary bile acids to secondary bile acids.[67]

Inhibition of intestinal FXR ameliorates high-fat diet induced hepatic steatosis in murine models.[68] Recent pre-clinical studies have also supported a role for microbial alterations in bile acid metabolism, specifically an overabundance of deoxycholic acid (a secondary bile acid), in the development of obesity-related liver cancer.[69] However, it is imperative to highlight that differences in bile acid composition between humans and mice must considered when extrapolating these findings to humans.[70]

FXR agonists have been studied in the management of NASH in humans. A clinical trial of obeticholic acid, a potent FXR agonist, in patients with non-cirrhotic NASH showed that 72-week treatment improved histological NASH.[71] Adults with NAFLD have elevated total serum bile acids and alterations in the ratio of secondary to primary bile acids[72], which was subsequently found to be associated with a shift in abundance of gut microbes associated with bile acid deconjugation.[73] Moreover, fecal bile acid composition distinguishes adults with NASH from healthy controls.[13] (Table 1) Overall, while it is likely that altered bile acid metabolism and ensuing effects on FXR signaling contribute to NAFLD pathogenesis, there is still much to be gleaned from the complex, reciprocal relationship between the gut microbiome and bile acids.

5. Ethanol production

Alcohol is mainly metabolized in the liver by alcohol dehydrogenase (ADH), and hepatic gene transcription of ADH is increased in NAFLD.[74] Ethanol production in the gut can be altered by manipulation of the gut microbiome[75], and a common finding in multiple NAFLD phenotypes is enrichment in *Escherichia* and *Lactobacillus*, genera which can produce ethanol.[2] (Table 1) While increased serum and fecal ethanol has been described pediatric patients with NAFL[8] and NASH[7], this finding has not been replicated in adult cohorts.[18] Recent evidence suggests that insulin-dependent impairments of liver ADH activity, as opposed to endogenous alcohol synthesis from the gut microbiome, could explain elevated serum ethanol levels in NAFLD, pointing to the existence of distinct disease mechanisms that may be related to differences in gut microbiome function.[76] Ultimately, additional studies are needed to confirm these findings.

6. Amino acid biosynthesis and metabolism

The gut microbiome can exert effects on amino acid homeostasis, in part due to biosynthesis and metabolism of aromatic (AAA) and branched-chain amino acids (BCAA). Cohort studies have identified elevated serum BCAAs as a biomarker for insulin resistance.[77, 78] In adults with insulin resistance, *Prevotella copri* and *Bacteroides vulgatus* are associated with enriched biosynthetic potential for BCAAs and a reduced potential for BCAA transport into bacterial cells.[28]

Perturbations in microbial metabolism of AAAs and BCAAs, and ensuing alterations in the serum metabolite profile, have more recently been identified in adults with NAFL.[21] (Table 1) Phenylacetic acid, an AAA-derived microbial metabolite strongly correlated with hepatic

steatosis in humans, was found to induce hepatic steatosis in both a primary culture of human hepatocytes and in rodents, suggesting a causal role in NAFL pathogenesis.[21] This study provides a proof-of-concept of how findings from integrated multi-omic analyses in patient cohorts can be complemented by *in vitro* and *in vivo* mechanistic studies, in order to facilitate the identification of microbial-driven causal pathways.

The gut microbiome and NAFLD: from research to bedside—Microbiome-targeted therapy (MTT) is considered to include antibiotics, probiotics (culture of living microorganisms which could have health benefits for the human host), prebiotics (fermentable dietary fibers that stimulate the growth and survival of probiotics), synbiotics (combination of probiotics and prebiotics), and FMT.[1] High-quality, large-scale clinical interventional trials examining MTT in NAFLD are lacking. Several randomized controlled trials have examined the use of non-FMT MTT in NAFLD but yielded mixed results.[79] To date, FMT trials in human subjects have been limited to obese adults with metabolic syndrome (without defined NAFLD), but at least two clinical trials examining FMT in adults with biopsy-confirmed NASH are actively recruiting subjects.[81, 82] FMT from lean to obese donors was shown to improve insulin sensitivity, albeit with only short-term improvement.[63, 80] Given NAFLD is commonly associated with insulin resistance, these results suggest that FMT could be efficacious in the management of NAFLD, however, improvement may be short-lived and limit the feasibility of this approach.

Ultimately, until we better understand the intricacies of microbial metabolism and microbe-host interactions in the pathogenesis of NAFLD, it's unlikely that MTT will be optimized for use in clinical practice. Nonetheless, even without elucidation of exact mechanistic pathways, stool and/or serum microbial biomarkers will likely yield diagnostic and/or prognostic utility. This is especially relevant in NAFLD given the need for non-invasive approaches to evaluate for progressive forms of disease.

Conclusions

In summary, pre-clinical evidence supports a causal role of gut microbiome in liver disease progression in NAFLD. However, there is much that we do not understand about the gut-liver axis. Due to the cross-sectional nature of published human data, along with methods utilized for microbial profiling, the majority of clinical evidence supports an association between dysbiosis and NAFLD, but mechanistic links have not been well established. Further well-designed, longitudinal, prospective cohort studies with multi-omic profiling techniques, and complemented by mechanistic studies in animal models, are needed to decipher the complex (and likely multifactorial) microbiome-host interactions in NAFLD. Nevertheless, it is becoming increasingly apparent that the gut microbiome may play a role as a biomarker of disease severity in NAFLD and provide novel insights into the pathogenesis of NAFLD in the coming years.

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Abbreviations:

(ADH)	alcohol dehydrogenase
(AAA)	aromatic amino acids
(BMI)	body mass index
(BCAAs)	branched-chain amino acids
(DNA)	deoxyribonucleic acid
(FXR)	farnesoid X receptor
(FMT)	fecal microbiota transplantation
(HCC)	hepatocellular carcinoma
(LPS)	lipopolysaccharide
(MTT)	microbiome-targeted therapy
(NAFL)	nonalcoholic fatty liver
(NAFLD)	nonalcoholic fatty liver disease
(NASH)	nonalcoholic steatohepatitis
(rRNA)	ribosomal ribonucleic acid
(SCFA)	short-chain fatty acid
(SNP)	single-nucleotide polymorphism
(TLR4)	toll-like receptor 4
(TMA)	trimethylamine
(TMAO)	trimethylamine-*N*-oxide

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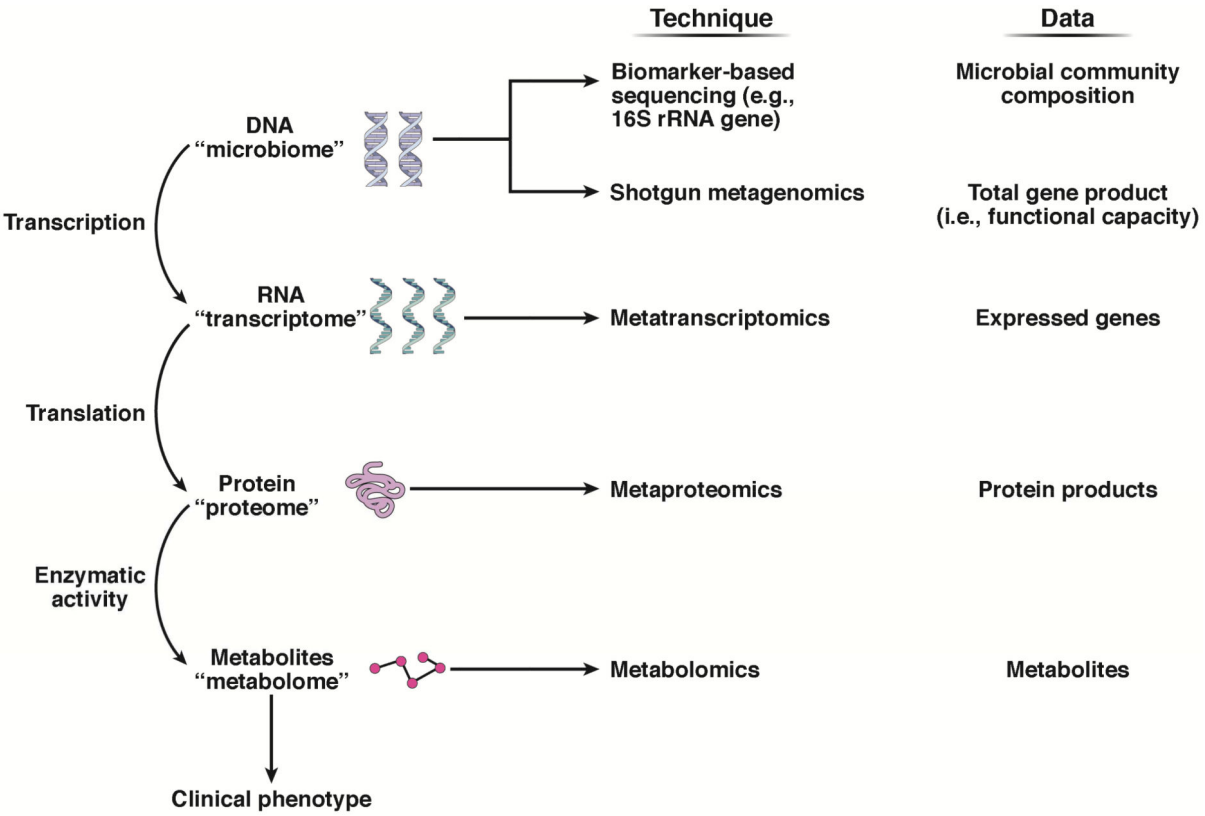

Figure 1.
Methods for characterization of the gut microbiome.

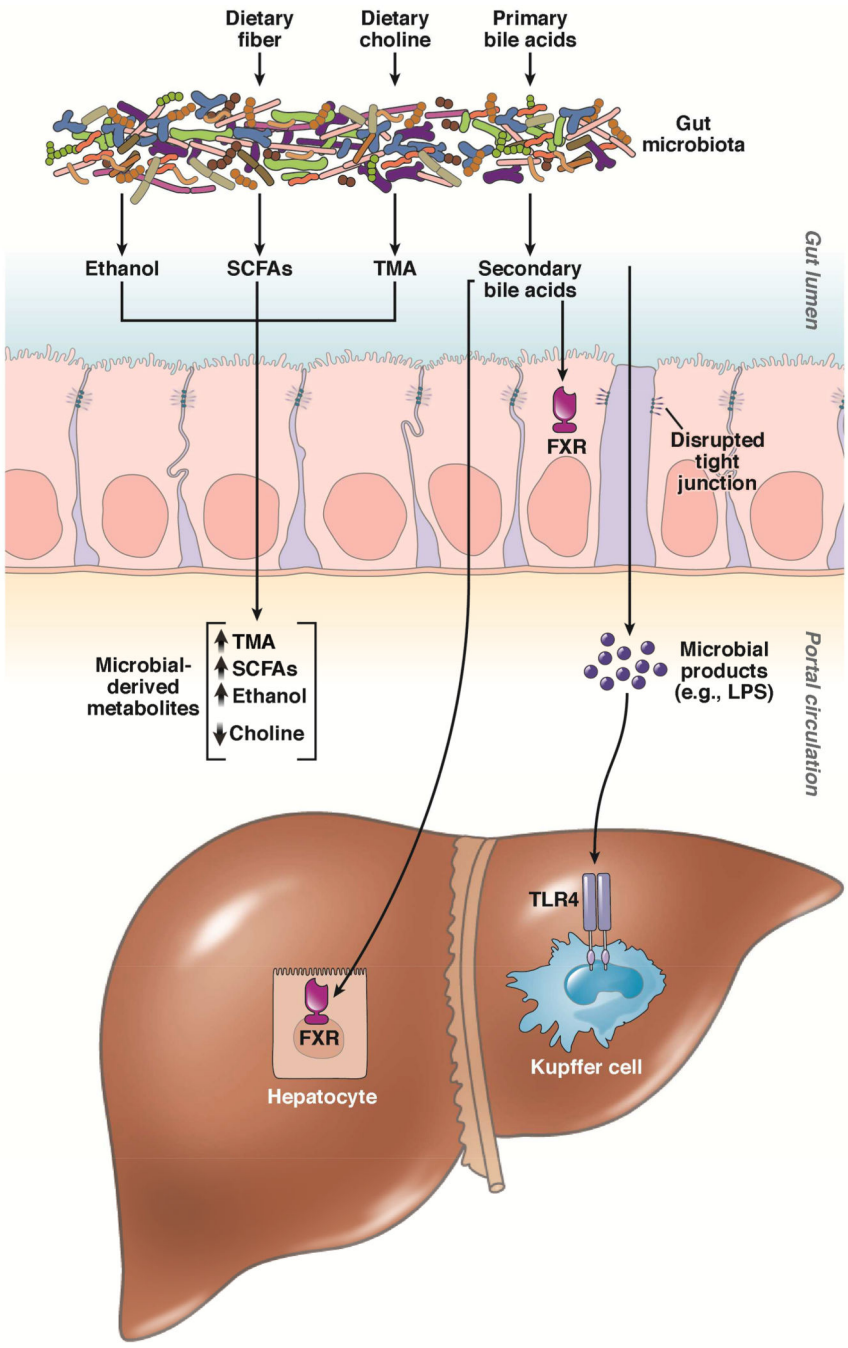

Figure 2. Postulated mechanisms linking the gut microbiome to nonalcoholic fatty liver disease.

Impairment of intestinal epithelial function, via disrupted tight junctions, leads to endotoxemia and induction of toll-like receptor 4 (TLR4). Dietary choline is metabolized by the gut microbiome to produce trimethylamine (TMA), which reduces choline bioavailability. Indigestable carbohydrates (e.g. dietary fiber) undergo fermentation by the gut microbiome and give rise to short-chain fatty acids (SCFAs). The gut microbiome metabolizes bile acids, thus regulating the bile acid pool and leading to alterations in

farnesoid X receptor (FXR) signaling. Ethanol can be generated by the gut microbiome. The gut microbiome may contribute to disruptions in amino acid homeostasis (not pictured).

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Table 1.

Characterization of the gut microbiome in clinical phenotypes of nonalcoholic fatty liver disease (NAFLD), stratified by genus-level taxonomic composition and "functional potential"

Clinical Phenotypes of NAFLD	Community Composition*		Functional Potential+	
	Phylum	Genus	Fecal Metabolites	Serum Metabolites
NAFL±	Firmicutes	↑*Blautia*[16] ↑*Dorea*[11] ↓↑*Roseburia*[15;11] ↓↑*Lactobacillus*[15,11,10,18,23] ↑*Streptococcus*[23] ↑*Clostridium*[23] ↓*Oscillospira*[9] ↓*Coprococcus*[21,18,15] ↓*Faecalibacterium*[18] ↓*Moryella*[15] ↓*Flavonifractor*[19] ↓↑*Oscillobacter*[11,23,15;21] ↓↑*Ruminococcus*[18,16,15;9]	↑Butanoic acid[11] ↑Propanoic acid[11] ↑Acetic acid[11] ↑Isobutyric acid[18] ↑Propionate[18] ↑Unconjugated cholic acid[13] ↑Ethanol[8] ↓2-butanone[11]	↑2-butanone[9] ↑1 pentanol[9] ↑2-hydroxy-butyrate[18] ↑L-lactic acid[18] ↑Phenylacetic acid[21] ↑Valine[21] ↑Leucine[21] ↑Isoleucine[21]
	Bacteroidetes	↓↑*Prevotella*[16,23;8] ↓*Odoribacter*[23] ↓*Alistipes*[23]		
	Proteobacteria	↓*Escherichia*[21,16,23]		
	Actinobacteria	↓↑*Bifidobacterium*[10;21]		
NASH±	Firmicutes	↑*Blautia*[9,16] ↑*Dorea*[9] ↑*Lactobacillus*[10,19] ↑*Clostridium*[13] ↑*Allisonella*[12] ↑*Oscillospira*[9] ↓*Coprococcus*[18] ↓*Faecalibacterium*[12,18,19] ↓↑*Ruminococcus*[18,19,9]	↑Chenodeoxycholic acid[13] ↓Unconjugated cholic acid[13] ↑Lithocholic acid[13]	↑Ethanol[7] ↑2-butanone[9] ↑4-methyl-2-pentanone[9]
	Bacteroidetes	↑*Bacteroides*[24] ↑*Parabacteroides*[12] ↓*Prevotella*[24]		
	Proteobacteria	↑*Escherichia*[7]		
	Actinobacteria	↓*Bifidobacterium*[10,19]		

Clinical Phenotypes of NAFLD	Community Composition*		Functional Potential+	
	Phylum	Genus	Fecal Metabolites	Serum Metabolites
NAFLD-related advanced fibrosis	Firmicutes Bacteroidetes Proteobacteria	↑*Blautia*[20] ↑*Roseburia*[20] ↑*Streptococcus*[20] ↑*Lactobacillus*[20] ↓↑*Enterococcus*[20] ↑*Ruminococcus*[17,20,24] ↑*Bacteroides*[20,17,22,24] ↑*Parabacteroides*[20] ↑*Escherichia*[17, 22,16] ↑*Klebsiella*[20] ↓↑*Prevotella*[24,20]		↑3 phenylpropanoate[17] ↑3-(4-hydroxyphenyl)lactate[22]
	Verrucomicrobia	↓*Akkermansia*[20]		
NAFLD-related HCC	Firmicutes	↑*Enterococcus*[20] ↑*Oscillospira*[20] ↓*Blautia*[20]		
	Bacteroidetes	↑*Bacteroides*[20]		
	Actinobacteria	↓*Bifidobacterium*[20]		

↑Increased in clinical phenotype of NAFLD; ↓Decreased in clinical phenotype of NAFLD; ↑↓Discordant findings among studies.

*Comparison groups differed amongst studies. In NAFL, comparison groups included healthy controls[9,8,10,11,15,16,18,23] and obese controls[21]. In NASH, comparison groups included healthy controls[9,10,12,13,18,19], obese controls[7], and NAFL[24,16]. In NAFLD-associated advanced fibrosis, comparison groups included healthy controls[20] and NAFLD without advanced fibrosis (stage<2)[17,24,22,16]. In NAFLD-related HCC, the comparison group included NAFLD-related cirrhosis without HCC[20].

+The fecal and serum metabolites listed are postulated to be derived from the gut microbiome.

±Two studies enrolled only non-obese subjects, in order to examine compositional changes in non-obese ("lean") NAFL[15] and NASH[19].

Abbreviations: nonalcoholic fatty liver disease (NAFLD), nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), and hepatocellular carcinoma (HCC).